IRON TOLERANCE ACROSS SEX AND BITING PROPENSITY IN *WYEOMYIA SMITHII* LARVAE

by

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A THESIS

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Mosquitoes are known as the most dangerous animals on earth because they transmit heinous diseases, including malaria, dengue, and yellow fever. Biting and sucking blood are hazardous to mosquitoes because, among other substances, blood contains toxic amounts of iron. This study aimed to determine whether iron tolerance varied between blood feeding and obligate non-biting populations of *Wyeomyia smithii*. Additionally, sex ratio was examined to seek out potential physiological differences between males and females between biting and non-biting populations. Using six serial dilutions of ferrous sulfate, I determined that sex does not alter survivorship to pupation nor adult emergence in biting nor non-biting populations. My research sought out the physiological limits of iron tolerance in larvae of the pitcher-plant mosquito, *W. smithii*, and discovered significantly decreased frequency to pupation across all populations at high concentrations of ferrous sulfate. Through the discovery of this threshold level of iron, my results provide an important step for future research into adult iron tolerance in *W. smithii*. In the adult life stage, both females and males feed on carbohydrates and could be targeted with iron-laced sugar baited traps. Hence, my research will contribute to the control of mosquito borne disease using measures that are benign to the environment.

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Introduction

Mosquitoes have been called "the most deadly animals on Earth" (Armbruster, 2018, p. 1009) because they transmit pathogens causing some of the most lethal and debilitating human diseases worldwide. Mosquitoes are **vectors**[1] for diseases such as Malaria and Dengue, West Nile, Zika, and Chikungunya viruses (Bradshaw et al., 2017). In order for these diseases to be transmitted, the mosquito must take a blood meal (hereafter, bite) from an individual host. Blood feeding results in a massive intake of iron due to the breakdown of **hemoglobin** during blood digestion. Unless sequestered or rapidly excreted, iron is toxic to mosquitoes (Graça-Souza, 2006).

Only adult female mosquitoes bite. Females effectively sequester heme-bound iron in the **peritrophic matrix** lining their gut and, upon the breakdown of heme, rapidly excrete over 85% of the iron consumed from the bite (Zhou, 2007). Adult males do not bite, but they can imbibe fluid, and, if fed liquid blood, rapidly succumb from a toxic overload of iron (Nikbakhtzadeh, 2016). The internal physiology of digesting, sequestering, and excreting this heme-bound iron via the peritrophic matrix is better developed in adult females than adult males. Adult females that consume blood have a peritrophic matrix that grows in thickness as a direct response to blood consumption thereby protecting the body from heme-bound iron. Adult males and juveniles (larvae) of both sexes maintain a consistently thin peritrophic matrix (Lehane, 1997).

[1] Bolded terms are defined in the glossary at the end

While previous studies have considered the role of the peritrophic matrix in adult mosquitoes, no studies had evaluated iron toxicity in larval mosquitoes, much less larval differences between the sexes or among populations within a single species. My research set out to determine if iron tolerance varied in larval *Wyeomyia smithii* between males and females and among larvae from populations whose adult females bite or are obligate non-biters.

I found that iron sulfate is toxic to larval *W. smithii* through a decrease in frequency of pupation and emergence of adults as iron concentration experienced by larvae increased for all populations. Pupation and adult emergence was lowwer in non-biting than in biting populations throughout the concentration gradient. Sex ratio of pupae or emerging adults did not vary between biting and non-biting populations or with larval iron concentration.

Figure 1: *W. smithii* larva

Background

Wyeomyia smithii

The species of mosquito I examined, *Wyeomyia smithii*, lives in the water-filled leaves of the carnivorous purple pitcher plant, *Sarracenia purpurea*. The range of the mosquito follows that of its host plant across the eastern United States and westward in Canada, including both coastal and mountainous elevations (Bradshaw et al., 2017). *Wyeomyia smithii* is unique among all mosquitoes in that it bites in the southern part of its range and is obligate non-biting in the northern[2] part of its range; yet, all populations remain fully **inter-fertile** (Bradshaw et al., 2017).

Figure 2: Adult *W. smithii* inside *S. purpurea* leaf

[2] High elevation populations in North Carolina are evolutionarily grouped with northern populations (Mertz et al. 2013) and are obligate non-biters.

Materials and Methods

I tested six populations of *W. smithii*, three with the capability of taking a blood meal and three with obligate non-biting females. The wild populations were collected from Florida, Alabama, coastal and mountainous North Carolina, Maine, and Wisconsin in summer 2016.

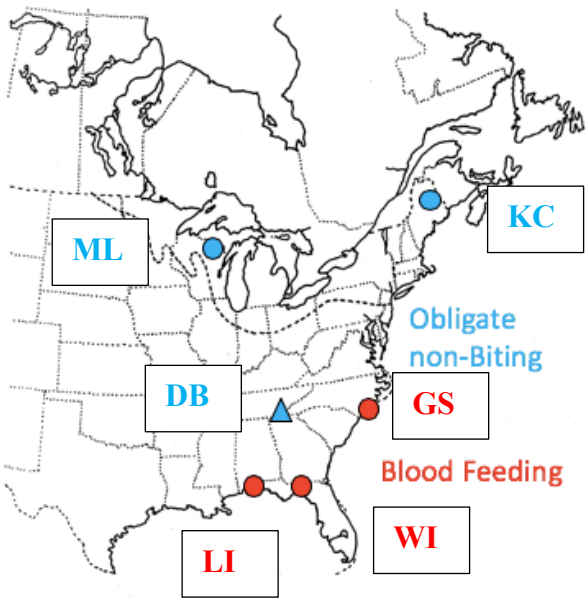

Figure 3. Map of experimental populations

Location	Name	State	N Latitude	W Longitude	m Elevation
DB	Dulaney Bg	NC	35.0	83.0	900
GS	Green Swmp	NC	35.1	78.3	20
KC	Knowles Crnr	ME	46.2	68.3	365
LI	Lilian	AL	30.4	87.5	15
ML	Muskelunge Lk	WI	46.0	89.6	500
WI	Wilma	FL	30.1	85.0	10

Laboratory Standard Rearing Procedure

Each population was isolated in separate crates with 35 larvae per 150 mm dish containing 1.08 x 10^{-1} g/L of tetracycline deionized water. Each dish was fed a 4:1 ratio of Geisler Guinea Pig Feed (Sergeant's Pet Care Products©) to freeze dried brine shrimp (San Francisco Bay Brand©). The mixture was ground and then sifted through a 0.50 mm sieve. The fine particles were then mixed with tetracycline deionized water and dispensed at 2 mL per dish weekly.

Larvae were raised in optimal short-day conditions receiving 8 hours of light per day and kept at a constant temperature of 21º C. To initiate pupation, dishes were moved into the optimal long day climate-controlled room receiving 16 hours of light per day, with constant 80% humidity, and set to a daily **thermoperiod** within a range of 15º C to 32º C. Pupa were collected into cups of 140 mL deionized water with no more than 50 pupa per cup. After 5 days as pupae, cups were placed in new enclosures. The bottom of the enclosures contained a layer of filter paper above layers of paper towels, all soaked in deionized water. The mesh tops supported a small handful of pesticide-free raisins to serve as the adult food source. Each enclosure contained one purple pitcher-plant leaf partially filled with deionized water. Paper was wet with deionized water and eggs were collected three times a week. Eggs hatched into deionized water in short-day conditions, before they were transferred to tetracycline deionized water with 35 larvae per dish.

Iron Solution Procedure

To make the six concentrations of iron solution, I used Nature Made © 325 mg tablets of ferrous sulfate incorporating 65 mg iron in each tablet. I made series of five dilutions (serial dilutions) of ferrous iron in addition to one control: 33.583 mg/L, 3.358 mg/L, 0.336 mg/L, 0.034 mg/L, and 0.003 mg/L. To do this, I dissolved 15.5 tablets of ferrous sulfate in 3 liters of larval antibiotic water. The tablets dissolved over at least a 24-hour period. After the tablets were dissolved, I measured 300 mL of this 335.833 mg/L solution into 2.7 L of antibiotic water. I repeated this process of measuring 300mL of each succeeding solution and mixing it into 2.7 L of antibiotic water, shaking each new concentration before transferring to the next dilution. This process was repeated five times resulting in five concentrations of ferrous iron from the initial 335.833 mg/L solution. All solutions were stored and sealed in one-gallon plastic jugs in a refrigerator at 3º C.

Parent Populations

To randomize the test populations, I separated out experimental dishes from laboratory stock population dishes, stored in the 21°C short-day room, using a histogram method. All dishes, containing 35 larvae per dish, were ordered by the date their eggs were collected in stacks, resembling a histogram. Total dishes were divided by 18 to calculate the pull number. Counting down the line of dishes, 18 dishes were counted out from each population and placed in the optimal long-day room. In the new climate conditions, the larvae continued to be raised with 2 mL of food solution per day. I switched larvae into new antibiotic water solutions and dispensed 2mL of food solution weekly. I collected pupae Monday, Wednesday, and Friday and raised them to

adulthood following standard procedures described above. The eggs collected from these six populations were placed into deionized water and labeled with population name and date of egg collection.

Experimental Procedures

Experimental eggs hatched in deionized water in short day conditions. After five days, I counted out 50 hatched larvae per 150 mm dish into 75 mL of their assigned solution in and feed 3 mL of food. Once in an experimental iron concentration, larvae were raised under the optimal long-day and temperature conditions to initiate pupation. I assigned iron concentration at random among the 84 dishes by turning cards, through the process of assigning suite and color to replicates. Each population, with two replicates of six iron sulfate concentrations, was switched and fed on a cycle alternating between 4 to 5 days starting from the initial solution date. I collected pupa Monday, Wednesday, Friday placed them in a closed cup with deionized water and labeled with population name, iron concentration, replicate number, and date. Pupa were checked Tuesday, Thursday, Saturday for adult emergence or death. I sexed the exuviae of adults and dead pupae under a microscope.

Statistical Approach

Prior to ANOVAs, frequencies were arcsin transformed to approximate a normal distribution. Arcsin-transformed frequencies of survivorship from hatch to pupation, survivorship from pupation to adult eclosion, and survivorship from hatch to adult eclosion, were subjected to two-way ANOVA with iron concentration, northern (non-biting) vs. southern (biting) populations, and their interaction used as fixed-effect

treatments. Populations within treatments were considered random effects; consequently, Mean Square biting *iron* population interaction was used as the error effect in F-tests for significance. ANOVAs were run using JMP (Sall, et al., 2005).

Due to low survivorship at high iron concentrations (Appendix I), sex of pupae or emerging adults were increasingly skewed towards zero or undefined when actually equal to zero. Consequently, 2x3 contingency tables were used to evaluate sex ratios of pupae and emerging adults at the zero and the two highest iron concentrations. χ^2 and associated *P*-values were calculated using the *Chisq test* function in Excel 2010. Correction for continuity was used only if the initial χ^2 indicated non-independence ($P<0.05$).

Results

Frequency of both pupation and adult emergence declined with increasing iron concentration and was lower among northern (non-biting) than southern (biting) populations. Southern populations did not achieve disproportionate pupation or emergence success at higher than lower iron concentrations as in neither case was there a significant interaction effect (Fig. 4A, 4C). Survivorship of pupae did not vary significantly with larval iron concentration, between biting and non-biting populations, or with their interactive effects (Fig. 4B).

Sex ratio of neither pupae nor emerging adults varied with iron concentration experienced by larvae or between non-biting and biting populations (Fig. 5).

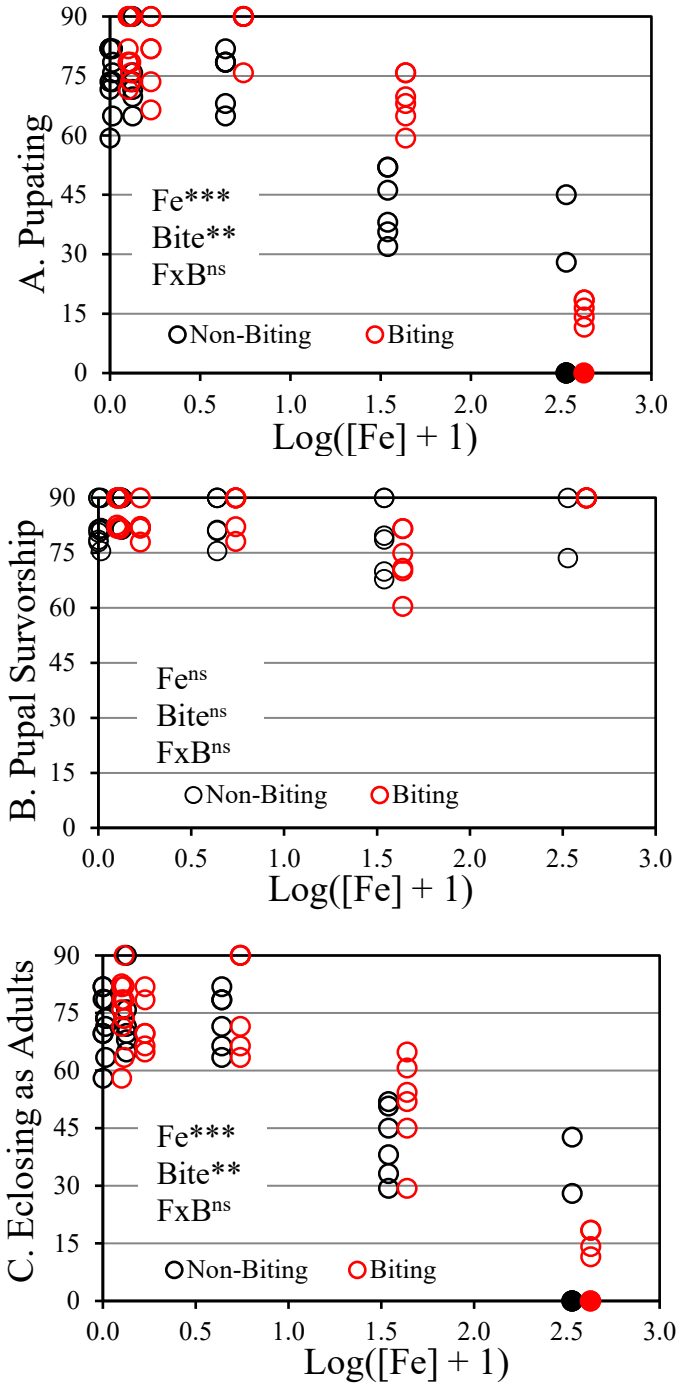

Figure 4. Survivorship to pupation (A), during pupation (B), or to adult emergence (C). In each plot, significance of two-way ANOVA (Appendix II) is given by the inset (Fe, iron concentration experienced by larvae; Bite, non-biting vs. biting populations; FxB, interaction between iron concentration and propensity to bite. ***P<0.001; **P<0.01; ns, P≥0.05). Arcsin-transformed values are shown on the vertical axis and Log10 iron concentration on the horizontal axis. The 1.0 is added to iron concentration to permit log-transformation of the zero control. The plots for biting populations are offset by 0.1 so that they can be visually compared with the corresponding plots for non-biting populations. Solid circles represent 2-4 of the populations with frequencies of zero.

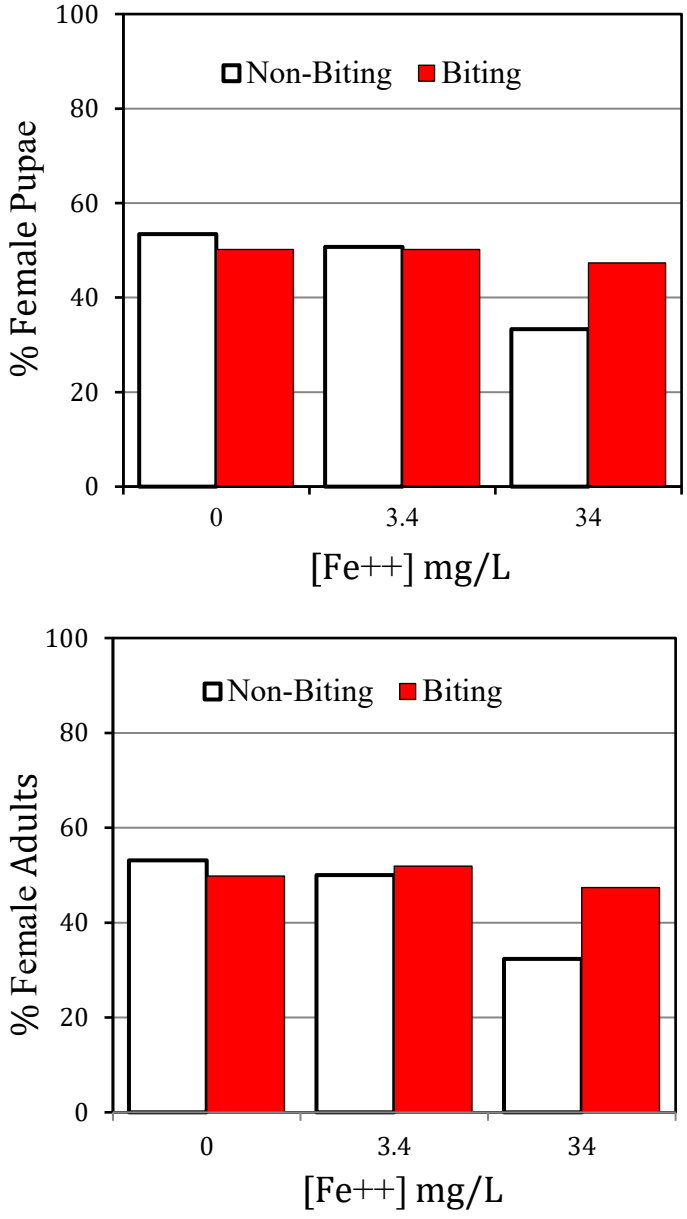

Figure 5. Sex ratio expressed as % female in non-biting and biting populations in response to the zero iron control and two highest iron concentrations among pupae (top) and eclosing adults (bottom).

	Pupae		Adults	
	North	South	North	South
χ^2	5.507	0.061	5.358	0.486
P	*0.076*	*0.971*	*0.073*	*0.855*

Table 1. Results of *Chisq test* for independence of sex ratio of pupae and emerging adults between obligate non-biting (North) and biting (South) populations (Appendix III).

Discussion

Through decreased frequency of *W. smithii* pupation and emerging adults in response to increased concentration of iron salts, my results show that the entire life cycle can be interrupted through the inability to process iron salt. I predicted variation of iron tolerance of larvae and a higher female-biased sex ration in southern populations with biting than in northern populations with obligate non-biting adult females. When exposed to toxic iron concentrations, frequency of pupation and emerging adults was in fact lower in the northern populations than the southern populations. However, sex ratio of larvae surviving to pupae or adults did not differ between northern and southern populations. Nontheless the differential toxicity between inter-fertile populations within a single species provides an entrée into determining genetic mechanisms of iron susceptibility in mosquitoes.

Wyeomyia smithii Larval Physiology

The peritrophic membrane of *W. smithii* larvae has previously never been studied. While my research does not directly examine this level of detail into the specific anatomical functions, the results do support the assumption that *W. smithii* fit into the broader knowledge of mosquito larval physiology. Previous research of generalized mosquito physiology found that juveniles (larvae) of both sexes maintain a consistently thin peritrophic matrix (Lehane, 1997). Since the sex ratio of pupae and emerging adults, regardless of the adult females' propensity to bite, did not vary in response to iron concentration, the larval peritrophic metrix is likely similar in both sexes of *W. smithii*, as in other larval mosquitoes (Lehane, 1997).

Environmentally Friendly Population Control

While larvae do not bite, my results provide an avenue towards a more environmentally friendly means of mosquito control. In addition to blood, mosquitoes also feed on carbohydrates and are attracted to sugar-baited traps. Iron could be added to the sugar bait along with substances that specifically interfere with iron tolerance in mosquitoes. By specifically targeting mosquito physiology, the impact on non-target species could be reduced. Without mosquitoes, there is no bite and therefore no disease transmission. Seeking to control mosquitoes with harsh chemicals not only can affect the ecosystem at large but also can impose risks to humans. Iron sulfate is naturally occurring and taken as a dietary supplement in humans. These qualities make it a promising option for mosquito population control.

Future Research

Finding a trend of low larval survivorship across all populations and both sexes at 33.583 mg/L of ferrous iron provides the first physiological limit for iron in *W. smithii*. This baseline toxicity level can be used to determine iron tolerance in the adult stage. Future research of adults would enhance the knowledge of iron toxicity to develop iron-laced sugar baits as an environmentally benign control measure. Through my study of the larval stage, the questions I investigated can be framed for adults: (1) Are avid blood-feeding mosquitoes more iron tolerant than reluctant or non-biting mosquitoes? (2) Does sex affect survivorship of adult *W. smithii* in response to increased iron concentrations?

Continued research can achieve the end goal of mosquito population-control to reduce the transmission of heinous diseases in a safe, environmentally responsible way.

Glossary

Eclosion: the process in which pupa emerge from their casing as fully formed adults

Hemoglobin: a complex oxygen-carrying protein incorporating heme, a complex of rings surrounding an iron molecule, which itself is the portion of hemoglobin actually carrying the oxygen.

Inter-fertile: different populations whose individuals are able to mate and produce living offspring

Peritrophic: literally, "around the food;" **matrix**: a semi-permeable, net-like structure that lines the gut and inhibits or prevents the ingested food or blood from contacting the actual gut lining itself.

Pupa: final state of reformation before adulthood in which larval structures rearrange to make the adult form within a casing

Pupation: the transition from the last stage of larval molting to become a pupa

Larva: first life stage after hatching from egg; individuals undergo four stages of molting called instars

Thermoperiod: fluctuating day and night temperature

Vector: an organism that carries and transmits a pathogen

Appendices

Appendix I. Collated data

					Asin				
#	Fe++ mg/L	Rpl	Biting	Pop	%P	%A	Psurv	%♀P	%♀A
CA	0.000	1	0	DB	82.0	78.6	78.6	45.6	44.4
CB	0.000	2	0	DB	73.6	69.7	78.0	46.2	46.3
6A	0.003	1	0	DB	81.9	78.5	81.8	39.7	40.2
6B	0.003	2	0	DB	78.5	71.6	75.5	48.6	48.2
5A	0.034	1	0	DB	73.6	71.6	81.5	43.8	43.1
5B	0.034	2	0	DB	75.8	75.8	90.0	38.2	38.2
4A	0.336	1	0	DB	81.9	81.9	90.0	43.2	43.2
4B	0.336	2	0	DB	78.5	78.5	90.0	47.4	47.4
3A	3.358	1	0	DB	51.9	51.9	90.0	38.5	38.5
3B	3.358	2	0	DB	51.9	50.8	79.7	45.9	45.0
2A	33.583	1	0	DB	45.0	42.7	73.6	36.9	36.1
2B	33.583	2	0	DB	28.0	28.0	90.0	31.5	31.5
CA	0.000	1	0	ML	81.9	81.9	90.0	51.5	51.5
CB	0.000	2	0	ML	71.6	69.7	81.4	46.9	46.3
6A	0.003	1	0	ML	.	.	.	.	.
6B	0.003	2	0	ML	73.6	73.6	90.0	45.0	45.0
5A	0.034	1	0	ML	90.0	90.0	90.0	39.2	39.2
5B	0.034	2	0	ML	69.7	68.0	81.3	46.3	45.7
4A	0.336	1	0	ML	78.5	71.6	75.5	45.0	45.6
4B	0.336	2	0	ML	78.5	78.5	90.0	40.2	40.2
3A	3.358	1	0	ML	35.7	33.2	69.9	43.3	39.2
3B	3.358	2	0	ML	38.1	38.1	90.0	46.5	46.5
2A	33.583	1	0	ML	0.0	0.0	0.0	0.0	0.0
2B	33.583	2	0	ML	0.0	0.0	0.0	0.0	0.0
CA	0.000	1	0	KC	81.87	81.87	90	45.58	45.58
CB	0.000	2	0	KC	59.34	58.05	80.54	45.77	46.59
6A	0.003	1	0	KC	64.9	63.43	81.02	47.1	47.87
6B	0.003	2	0	KC	75.82	73.57	81.61	38.23	38.72
5A	0.034	1	0	KC	64.9	64.9	90	44.3	44.3
5B	0.034	2	0	KC	71.57	69.73	81.43	39.23	39.76
4A	0.336	1	0	KC	68.03	66.42	81.23	55.21	56.2
4B	0.336	2	0	KC	64.9	63.43	81.02	41.5	42.13
3A	3.358	1	0	KC	31.95	29.33	67.79	57.69	60

3B	3.358	2	0	KC	46.15	45	78.69	47.21	48.45
2A	33.583	1	0	KC	0	0	.	.	.
2B	33.583	2	0	KC	0	0	.	.	.
CA	0.000	1	1	WI	71.57	71.57	90.00	40.53	40.53
CB	0.000	2	1	WI	90.00	82.58	82.58	48.83	48.41
6A	0.003	1	1	WI	90.00	90.00	90.00	50.03	50.03
6B	0.003	2	1	WI	90.00	82.03	82.03	46.10	46.69
5A	0.034	1	1	WI	81.87	78.46	81.79	44.42	43.81
5B	0.034	2	1	WI	90.00	81.87	81.87	39.23	39.71
4A	0.336	1	1	WI	90.00	90.00	90.00	39.92	39.92
4B	0.336	2	1	WI	90.00	90.00	90.00	48.96	48.96
3A	3.358	1	1	WI	59.34	54.33	70.80	42.67	42.39
3B	3.358	2	1	WI	68.03	60.67	70.06	38.96	40.46
2A	33.583	1	1	WI	18.43	18.43	90.00	39.23	39.23
2B	33.583	2	1	WI	11.54	11.54	90.00	90.00	90.00
CA	0.000	1	1	GS	78.46	75.82	81.70	42.61	41.95
CB	0.000	2	1	GS	78.46	78.46	90.00	41.41	41.41
6A	0.003	1	1	GS	78.46	78.46	90.00	42.61	42.61
6B	0.003	2	1	GS	73.57	73.57	90.00	46.25	46.25
5A	0.034	1	1	GS	66.42	66.42	90.00	49.11	49.11
5B	0.034	2	1	GS	73.57	69.73	77.96	45.00	43.70
4A	0.336	1	1	GS	90.00	90.00	90.00	36.87	36.87
4B	0.336	2	1	GS	75.82	71.57	78.10	50.52	50.77
3A	3.358	1	1	GS	69.73	64.90	74.86	46.30	47.10
3B	3.358	2	1	GS	64.90	51.94	60.41	45.70	49.64
2A	33.583	1	1	GS	14.18	14.18	90.00	35.26	35.26
2B	33.583	2	1	GS	18.43	18.43	90.00	26.57	26.57
CA	0.000	1	1	LI	90.00	90.00	90.00	47.61	47.61
CB	0.000	2	1	LI	81.87	81.87	90.00	47.93	47.93
6A	0.003	1	1	LI	75.82	73.57	81.61	38.23	38.72
6B	0.003	2	1	LI	73.57	71.57	81.52	43.75	43.09
5A	0.034	1	1	LI	90.00	82.25	82.25	40.29	40.74
5B	0.034	2	1	LI	81.87	78.46	81.79	46.76	46.19
4A	0.336	1	1	LI	90.00	82.18	82.18	41.81	42.29
4B	0.336	2	1	LI	90.00	90.00	90.00	48.94	48.94
3A	3.358	1	1	LI	75.82	73.57	81.61	46.83	47.49
3B	3.358	2	1	LI	75.82	73.57	81.61	49.28	48.75
2A	33.583	1	1	LI	16.43	16.43	90.00	60.00	60.00
2B	33.583	2	1	LI	0.00	0.00	.	.	.

Appedix II. Survivorship

Asin Sqrt % **Pupation**	DF	Sum of Squares	Mean Squares	F	df	P
Fe	5	42546.52	8509.30	66.359	5,10	2.42335E-07
Biting	1	1662.72	1662.72	12.966	1,10	0.005
Biting*Fe	5	1186.52	237.30	1.851	5,10	0.191
Fe*PopNum	10	745.08	74.51			
Biting*PopNum	2	598.09	299.05			
Biting*Fe*PopNum	10	1282.32	128.23			
Replicates	37	1821.76	52.05			
Total	70	50746.45				

Asin Sqrt % **Pupal Survivorship**	DF	Sum of Squares	Mean Squares	F	df	P
Fe	5	1537.30	307.46	0.940	5,10	0.496
Biting	1	613.17	613.17	1.874	1,10	0.201
Biting*Fe	5	1560.73	312.15	0.954	5,10	0.488
Fe*PopNum	10	3199.22	319.92			
Biting*PopNum	2	396.37	198.19			
Biting*Fe*PopNum	10	3271.81	327.18			
Replicates	34	6041.79	177.70			
Total	67	16620.39				

Asin Sqrt % **Adult Emergence**	DF	Sum of Squares	Mean Squares	F	df	P
Fe	5	40586.35	8117.27	68.26	5,10	2.11447E-07
Biting	1	1256.14	1256.14	10.56	1,10	0.009
Biting*Fe	5	845.43	169.09	1.42	5,10	0.297
Fe*PopNum	10	884.30	88.43			
Biting*PopNum	2	614.11	307.06			
Biting*Fe*PopNum	10	1189.12	118.91			
Error	35	1878.36	53.67			
Total	70	47961.68				

Appendix III. Sex ratio

Pupae

ΣNBf	**ΣNBm**	**ΣBf**	**ΣBm**
81	**68**	**71**	**77**
67	**61**	**82**	**75**
42	48	77	81
68	73	73	71
62	75	71	75
59	77	69	76
76	64	63	92
64	73	90	66
30	**32**	**65**	**63**
40	**36**	**65**	**66**
9	**16**	**6**	**6**
3	**8**	**3**	**4**

Adults

ΣNBf	**ΣNBm**	**ΣBf**	**ΣBm**
79	**68**	**70**	**77**
65	**59**	**81**	**75**
42	46	77	80
66	71	72	70
61	75	70	74
58	76	66	75
75	61	63	91
64	72	89	65
27	**31**	**62**	**58**
39	**35**	**60**	**55**
8	**15**	**6**	**6**
3	**8**	**3**	**4**

Pupal	NBf	NBm
0	148	129
3.4	70	68
34	12	24

Pupal	Bf	Bm
0	153	152
3.4	130	129
34	9	10

Adult	NBf	NBm
0	144	127
3.4	66	66
34	11	23

Adult	Bf	Bm
0	151	152
3.4	122	113
34	9	10

NB, non-biting; B, biting

f, females; m, males

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